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Synthesis and decay of calmodulin-ubiquitin conjugates in cell-free extracts of various rabbit tissues

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Abstract

Calmodulin is the natural substrate for ubiquitin-ligation by the enzyme ubiquitin-calmodulin ligase (uCaM-synthetase; EC 6.3.2.21). The activity of this ligase is regulated by the binding of the second messenger Ca^{2+} to the substrate calmodulin, which increases the activity ca. 10-fold. Up till now, two components of the ligase could be identified: uCaM Syn-F1 and uCaM Syn-F2, the first of which binds to ubiquitin and the second which binds to calmodulin. Since the physiological role of this enzyme is still unclear, this study was designed to examine whether the activity of uCaM-Synthetase in 40 000 × g tissue supernatants correlates with the calmodulin content in the various tissues. In reticulocytes, spleen, erythrocytes, testis and brain, which are rich in uCaM synthetase, the tissue contents calculated on the basis of activity measurements were between 4–80-fold higher than in red and white skeletal muscle. These activities did not correlate with the respective calmodulin contents of the tissues indicating that other factors were determining these enzyme levels. A second aim was to gain information on the role of the ATP-ubiquitin-dependent proteolytic pathway in those tissues displaying uCaM synthetase activity. In the reticulocyte system which contains the classical ATP-ubiquitin-dependent proteolytic pathway as measured with ^{125}I-BSA, no ubiquitin-dependent degradation of calmodulin could be detected. We therefore examined the other tissues of the rabbit with the substrate ^{125}I-BSA and succeeded in finding a ubiquitin-independent ATP-dependent proteolytic activity in every case but no ubiquitin-dependent activity. The ubiquitin-independent activity was highest in smooth muscle and red skeletal muscle being ca. 3–4-fold higher than in lung and testis. In 50% of the tissue crude extracts the time curve of calmodulin ubiquitylation progressed through a maximum indicating a dynamic steady state based on conjugate synthesis and decay. If a ubiquitylation pulse of 30 min was followed in liver crude extracts by the addition of EGTA, which specifically inhibits ubiquityl-calmodulin synthesis, a half-life of calmodulin-conjugate decay of 15–20 min is observed. A similar conjugate half-life of ca. 30 min was observed after addition of EDTA excluding

Abbreviations: *A*, absorption, optical density; BSA, bovine serum albumin; DTE, dithioerythritol; CaM, calmodulin; CaM-m, methylated calmodulin; BH-, Bolton-Hunter (e.g., BH-calmodulin); CT-, chloramine T (e.g., CT-ubiquitin); APF II, DEAE fraction II; FP-, fluphenazine (e.g., FP-test); SDS-PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; TCA, trichloroacetic acid; uCaM, ubiquityl-calmodulin; vol., volume; w/v, weight in g per volume; ww, wet weight; *Enzymes*: ATP-dependent-26-*S*-protease (26*S*-proteasome, EC 3.4.99); ATP-ubiquitin-dependent proteolytic pathway (ubiquitin protein ligase + ATP-dependent-26-*S*-protease); multicatalytic endopeptidase complex (20*S*-proteasome, EC 3.4.99.46); ubiquitin-calmodulin ligase (ubiquityl-calmodulin synthetase, EC 6.3.2.21); ubiquitin-protein ligase (E1, E2, E3; EC 6.3.2.19).

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that conjugate decay is due to an ATP-dependent proteolytic process. Studying the decay of purified ubiquitin-^{125}I-BH-calmodulin conjugates in cell-free reticulocyte extracts led to the discovery of an ATP-independent isopeptidase activity which splits ubiquitin-calmodulin conjugates without leading to detectable calmodulin fragments. The rapid decay of ubiquitin-calmodulin conjugates in tissue extracts can therefore be plausibly explained by a ubiquityl-calmodulin splitting isopeptidase activity. © 1997 Elsevier Science B.V.

Keywords: Calmodulin; Ubiquitin ligase; Ubiquityl-calmodulin synthetase; Ubiquityl-calmodulin isopeptidase; ATP-dependent proteolysis

1. Introduction

Ubiquitin calmodulin ligase (uCaM synthetase, EC 6.3.2.21) [1–3] was the second defined ubiquitin ligase system discovered. It is responsible for the Ca^{2+}-dependent conjugation of calmodulin with ubiquitin in eukaryotic organisms from yeast to mammals (for review see [4]) and the catalyzed reaction can be described according to the following equation (n is an integer):

$$\text{calmodulin} + n\,\text{ubiquitin} + n\,\text{ATP} \overset{Ca^{2+}}{\rightleftharpoons} (\text{ubiquitin})_n - \text{calmodulin} + n\,\text{AMP} + n\,\text{PPi} \quad (1)$$

Experiments in vitro suggest that ubiquitylation decreases the biological activity of the Ca^{2+}-regulator protein [4,5].

The calmodulin ubiquitylating enzyme system (Eq. (1)) can be differentiated from the fairly unspecific ubiquitin-protein ligase system (EC 6.3.2.19; for reviews see [4,6]) which typically catalyzes the ubiquitylation of various artificial protein substrates in reticulocytes [7,8] according to the *N*-end rule (ATP-ubiquitin-dependent proteolytic pathway). Besides the ATP-ubiquitin-dependent proteolytic system, which contains the ATP-dependent-26-*S*-protease, other ubiquitin-independent systems have also been described [9–13]. An ATP-dependent protease of this type from cardiac muscle which we described [11] has a molecular mass of 310–350 kDa [11,14], is ubiquitin-independent, and can be differentiated [14] from the other major higher molecular mass proteases (for reviews see [15,16]) such as the 20S multicatalytic-endopeptidase-complex (EC 3.4.99.46) and the ATP-dependent-26-*S*-protease.

As yet little is known about the distribution of ubiquitin ligases in general and the different ATP-dependent proteolytic activities in mammalian tissues. In addition the mechanism and regulation of the physiological degradation of intracellular proteins under steady state conditions is still a major enigma [4].

In this paper the specific Ca^{2+}-dependent *ubiquitin-calmodulin ligase* and the *ATP-dependent proteolytic activity* (ubiquitin dependent and independent) as measured with the classical artificial substrate ^{125}I-BSA [17–20] were determined in various rabbit tissues. Both the ligase and an ATP-dependent proteolytic activities could be detected in all organs tested. In many cases the synthesized ubiquitin-calmodulin conjugates exhibited a dynamic state, resulting from a strong opposing reaction to ubiquityl-calmodulin synthesis. Evidence is presented which suggests that this opposing reaction is due to isopeptidase activity.

2. Materials

Ubiquitin, phenylhydrazine and Fluphenazine were purchased from Sigma (Munich). Creatine kinase (EC 2.7.3.2) was from Boehringer (Mannheim). Leupeptin and dithioerythritol (DTE) were obtained from Biomol (Hamburg). Coomassie Brilliant Blue R-250 (= Serva Blue R), BSA, deoxyglucose and 2,4 dinitrophenol were obtained from Serva (Heidelberg). Evipan (hexobarbital) was obtained from Bayer AG (Leverkusen). Silicone spray was obtained from Rüsch AG (Kernen-Rommelshausen). The molecular weight standards for SDS-PAGE bovine serum albumin (BSA) 66 kDa, ovalbumin 45 kDa, glyceraldehyde 3-phosphate dehydrogenase 36 kDa, carbonic anhydratase 29.2 kDa, trypsinogen 25 kDa, trypsin inhibitor 20.1 kDa and lactalbumin 14.2 kDa were obtained from Sigma (Munich). Sepharose 6B and DEAE-Sephacel were purchased from Pharmacia (Freiburg). XM100 ultrafilters for pressure filtration on Amicon cells were obtained from Amicon (Witten). Enhancer foils (Cronex Lightning Plus) were

obtained from Dupont (Neu Isenburg). X-ray films (Fuji RX, NIF) were obtained from Fuji (Düsseldorf). All chemicals were of the highest available or analytical grade. Water was deionized, distilled and then passed through a Milli-Q-system (Millipore, Witten) before use.

3. Methods

3.1. Preparative methods

3.1.1. Reticulocytes

Reticulocyte-rich (ca. 85%) blood was generated in rabbits (3–4 kg, obtained from a local dealer) by the phenylhydrazine method [21] as described previously [11]. In this procedure, 2.5% (w/v) phenylhydrazine in 0.9% NaCl, pH 7.4, is injected s.c. into the back of rabbits. On days 1–4 an amount of 0.22 ml/kg and on day 5 an amount of 0.13 ml/kg was injected. On day 8 rabbits were given a lethal overdose of 2.5 ml Evipan (100 mg/ml) and bled for harvesting of reticulocytes by incision of the jugulars. Coagulation was inhibited by addition of 1 M sodium citrate, pH 7.4, to a final concentration of 10 mM into polyethylene beakers coated with silicone spray. All further work was performed with beakers and tubes siliconized in this way. The reticulocytes were washed twice at 5°C in a 10-fold volume of buffer containing 10 mM potassium phosphate, 0.15 M NaCl, pH 7.4 (= buffer A).

3.1.2. Reticulocyte and erythrocyte lysates.

The washed reticulocytes were depleted of ATP as previously described [11] by incubation in 10 mM 2-deoxyglucose and 0.1 mM 2,4-dinitrophenol in buffer A for 1 h at 37°C [22] and were then washed twice in buffer A. Lysis was obtained by addition of 1.5 vol 1.66 mM DTE to the packed cells. The lysed fraction was centrifuged for 90 min at $80\,000 \times g$ to remove particulate and membranous material and stored frozen at −80°C (= reticulocyte lysate). Erythrocyte lysate was prepared in a similar manner except that the cells were washed 3 times and ATP-depletion step was omitted.

3.1.3. Tissue extracts

Rabbits were given a lethal overdose of Evipan (see above) and immediately exsanguinated by decapitation and suspending from the hind legs. The excised tissues were extensively washed with water to remove residual blood. Rabbit hearts were dissected to remove the residual blood in the heart chambers. The excised tissue was cut into small pieces and immediately frozen in liquid nitrogen. The frozen tissue was then transferred to a −80°C freezer and stored. For the described experiments the tissues of five rabbits were pooled. In control experiments (cardiac muscle, reticulocytes) the activities in fresh and frozen tissues were compared and found to yield equal results (not shown). The preparation of tissue extracts is based on the previously described procedure [11]. According to this method 6 g of frozen (−80°C) rabbit tissue (pooled from 5 rabbits) was homogenized in 3 mM potassium phosphate, 5 mM EDTA, 1 mM DTE, 5 μg/ml leupeptin, pH 7.4 (buffer B) in a Bühler homogenizer (E. Bühler, Bodelshausen) at full speed for 1 min at 4°C. This homogenate was centrifuged at $40\,000 \times g$ for 30 min in a Beckman L-7/80 ultracentrifuge and the supernatant (= crude extract) was collected as previously described in Ref. [11]. In contrast to the intact tissues the tissue crude extracts could not be stored frozen. Therefore only activities measured in crude extracts freshly prepared from frozen tissues are shown.

3.1.4. DEAE-Sephacel chromatography

The DEAE-cellulose method employed for the enrichment of the tissue extracts and previously described in Ref. [11,23] is based on the method of preparing the ATP-dependent proteolytic fraction II [3,20,22] also called APF II. In this step, proteins like hemoglobin, myoglobin and especially ubiquitin (see Ref. [22]) run unadsorbed through the column. Ubiquitin conjugating activity including uCaM-synthetase [20], calmodulin and many other proteins are adsorbed, enriched and then desorbed with KCl (see below). APF II pools were stored frozen at −80°C in aliquots. In all cases the activities in stored APF II pools were measured within 24 h and aliquots were thawed only once.

3.1.4.1. Reticulocyte lysate. 14 ml of Iysate (40–50 mg/ml) was fractionated on 11 ml DEAE cellulose (DEAE-Sephacel, Pharmacia, Freiburg, 1.4 cm i.d. × 7.2 cm gel height) in 3 mM KH_2PO_4/K_2HPO_4, 1 mM DTE, pH 7.0 (buffer C) [11]. The total adsorbed

protein which is step-eluted (vol: 25–35 ml) by addition of 500 mM KCl [11,22] to buffer D (20 mM Tris/HCl, 1 mM DTE, 5 μg/ml leupeptin, pH 7.2) was concentrated 4–8-fold by ammonium-sulfate precipitation (90% saturation) and dialyzed against 20 mM Tris/HCl, 1 mM DTE, 5 μg/ml leupeptin, pH 7.6 (buffer E) yielding reticulocyte APF II.

3.1.4.2. Tissue extracts and erythrocyte lysate. 10–15 ml of tissue crude extracts derived from 6 g of homogenized tissue (10–30 mg/ml; exception eye lens: 106 mg/ml) or 14 ml erythrocyte lysate (35 mg/ml) were applied to 4 ml DEAE-Sephacel columns (1.4 cm i.d. $\times$ 2.6 cm gel height) in buffer C and then after washing protein was desorbed in one step by increasing the KCl concentration to 350 mM [3,11,20,23]. The eluate pools (16–25 ml) were pressure concentrated in Amicon cells on XM 100 filters to 1/3–1/5 of the original volume (= tissue APF II). The run-through contained maximally ca. 10% of the applied enzyme activities except for uterus (35%) and spleen (25%).

3.1.5. Ubiquitin

Ubiquitin was prepared according to [11] or purchased from Sigma (Munich). Both the self-prepared and the commercial ubiquitins were tested for biological activity. The ubiquitin concentration necessary for half-maximal activation of ATP-dependent proteolysis as tested with reticulocyte APF II was 1.5–2 μM (see Ref. [11]). Ubiquitin$_t$ (= des-gly-gly ubiquitin), which cannot be conjugated to ubiquitin was prepared according to [24]. For the preparation of ubiquitin$_t$ reaction mixtures contained in a total volume of 650 μl: 37 mg/ml ubiquitin, 0.37 mg/ml trypsin, 50 mM NH_4HCO_3/ pH 7.8, 50 mM NaCl and 2 mM Ca^{2+}. The reaction was stopped after 30 min by adding 120 μl trypsin inhibitor (10 mg/ml). The formed ubiquitin$_t$ was dialyzed in Centricon 10 tubes against 20 mM Tris/pH 7.5, 1 mM DTE and stored frozen at -80°C. ^{125}I-labeled ubiquitin was synthesized according to the chloramine-T procedure (i.e. ^{125}I-CT-ubiquitin, 0.1–8 $\cdot$ 10^8 cpm/mg) [11,25].

3.1.6. Calmodulin

Bovine testis calmodulin was prepared as described [26]. The concentration of calmodulin necessary for half-maximal activation of phosphorylase kinase (maximal activation 6–7-fold) was 30–70 nM [26]. Unlabeled and ^{125}I-labeled BH-calmodulin, which is ubiquitylated at similar rates as native calmodulin [27]) was synthesized according to the Bolton and Hunter method [27,28]. ^{125}I-labeled BH-calmodulin was methylated according to the method described for ubiquitin [27,29,30]. Fluphenazine-Sepharose (FP-Sepharose) was prepared from Fluphenazine and Sepharose 6B as described in [26] and employed for the purification of calmodulin and for assaying uCaM-synthetase (see below).

3.1.7. Ubiquityl-calmodulin

Ubiquitin conjugates of calmodulin were prepared in analogy to the FP-test [2]. Reaction mixtures (10 ml) contained 50 mM Tris/HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 10 mM phosphocreatine, 1.1 mM $CaCl_2$, 1 mM EGTA, 0.1 mg/ml creatine kinase, 3.0 mg/ml ubiquitin, 0.5 mg/ml ^{125}I-BH-calmodulin (specific radioactivity 25–440 $\times$ 10^8 cpm/mg) and 3.6 mg/ml APF II at pH 8.0. The reaction was stopped after 4 h at 37°C by adding 30 ml of 20 mM sodium β-glycerophosphate, 1 mM $CaCl_2$, pH 7.0 and heating to 96°C for 10 min. Subsequently the mixture was placed on ice for 5 min. Denatured proteins were spun down (20 000 $\times$ g; 15 min; 4°C) and the supernatant was applied at room temperature to a Fluphenazine column (1.5 cm i.d. $\times$ 2.8 cm gel height; 5 ml packed gel) equilibrated with 20 mM sodium β-glycerophosphate, 1 mM $CaCl_2$, pH 7.0 (buffer F). The column was washed with buffer F containing 300 mM NaCl and eluted with 20 mM sodium β-glycerophosphate, 10 mM EGTA, 500 mM NaCl, pH 7.0. The eluate was concentrated by TCA precipitation (5%), the resulting pellet was neutralized with 1M sodium phosphate and resuspended in 20 mM sodium phosphate, 1 M NaCl, pH 7.0.

The conjugates were then adsorbed to a column of Chelating Sepharose Fast Flow (Fa. Merck, Darmstadt) (1.5 cm i.d. $\times$ 2.8 cm gel height) charged with Cu^{2+} and equilibrated with 20 mM sodium phosphate, 1 M NaCl, pH 7.0. Elution of the ubiquityl-calmodulin conjugates was done by a series of acetate buffers adjusted to different pH-values. Ubiquityl-calmodulin conjugates were eluted at pH 3.2, concentrated and dialyzed in Centricon 10 tubes against 10 mM sodium β-glycerophosphate, 0.1 mM $CaCl_2$, pH 7.0.

Table 1
Activity of ubiquityl-calmodulin synthetase in crude extracts of rabbit tissues [c]

Tissue	Protein	uCaM-synthetase				Calmodulin	Ratio
		Activity		Maximum type curve	Approx. tissue contents	Approx. tissue contents [38]	$\frac{\text{synthetase}}{\text{calmodulin}}$
	(mg/ml)	(fkat/ml)	(fkat/mg)		(fkat/g ww)	(μg/g)	fkat/μg CaM
Reticulocytes [a]	34.1	467.2 ± 21.8	13.7	−	2339	120 [b]	19.5
Spleen	20.3	430.4 ± 11.2	21.2	+ +	933	80	11.7
Kidney	19.7	116.2 ± 12.0	5.9	+	306	90	3.4
Erythrocytes [a]	35.0	49.0 ± 4.2	1.4	−	239	120	2.0
Testis	10.9	82.8 ± 7.0	7.6	+	239	450	0.5
Brain (cerebrum)	10.4	117.9 ± 10.8	11.3	+	217	500	0.4
Uterine smooth muscle	12.1	78.7 ± 22.3	6.5	+ +	190	240	1.3
Lung	27.5	85.3 ± 5.8	3.1	−	187	140	1.4
Cardiac muscle	10.3	73.1 ± 3.0	7.1	+ +	137	40	3.4
Liver	31.0	65.1 ± 5.6	2.1	+ +	130	80	1.6
Red skeletal muscle	12.7	27.9 ± 1.3	2.2	−	52	NG	
Eye lens	106.0	27.6 ± 1.5	0.3	−	37	NG	
White skeletal muscle	17.4	14.8 ± 1.2	0.9	−	27	38	0.7

[a] fkat/g packed cells ($3000 \times g$) not per g wet wt.
[b] Erythrocyte value, own values estimated from immunoblots of APF II were 60 μg/g.
[c] The incorporation of ^{125}I-ubiquitin into calmodulin (CaM) was measured with the FP-test (see Section 3 and Refs. [2,29]). The incubation mixture contained in a final volume of 100 μl: 50 mM Tris/HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 10 mM phosphocreatin, 0.1 mM $CaCl_2$, 0.1 mg/ml creatin kinase, 0.5 mg/ml calmodulin and 0.05 mg/ml ubiquitin ($7.6 \cdot 10^8$ cpm/mg). Controls were run without calmodulin. The activity (±SE) was calculated from the linear portions of activity progress curves (see Fig. 1) by linear regression analysis ($n = 5$ time points including origin) and corresponds to the conjugation of exogenous calmodulin. A similar percentual error applies to the derived quantities (i.e. specific activity and organ contents). The protein concentration of the assay mixture was 1/3 of the values given. In the case of reticulocytes the concentration was 6.4 mg/ml. (+) indicates a maximum-type curve, (−) indicates a plateau-type curve (see Fig. 1). The ratio in the last column is formed from the synthetase tissue content/calmodulin tissue content. For further details see Section 3 and text. NG, not given.

3.2. Analytical methods

3.2.1. Enzyme activities

As described above crude tissue extracts and APF II were prepared from the pooled tissues of 5 rabbits. In all cases the preparation of extracts and APFII was repeated independently at least once except for brain, eye lens and uterus, which were obtained in too low amounts. The enzyme activities measured in two such independent preparations showed a good comparability (uncertainty ca. 30%). In the tissue distribution studies (Tables 1–4) the enzyme activities were determined in identical tissue samples. The enriched fractions (Tables 2 and 4) were prepared from the respective same extracts shown in Tables 1 and 3.

3.2.1.1. ATP / Mg^{2+}-dependent protease(s) [11]. Substrate in this procedure is ^{125}I-labeled bovine serum albumin (^{125}I-BSA). An amount of 25 μg bovine serum albumin (BSA) was iodinated according to ref. [31], mixed with 2.5 mg BSA, acetylated according to ref. [24] and dialyzed against buffer G (20 mM Tris/HCl, pH 7.8). It was then precipitated by 5% w/v trichloroacetic acid (TCA). The pellet was taken up in 300 μl 1.0 M NaOH, diluted to a total volume of 6.0 ml in water, heated to 60°C for 15 min and stored frozen at −80°C (^{125}I-BSA stock solution). Before use the stock solution was diluted 10–15-fold in 50 mM Tris/HCl, pH 8.0. Methylated ^{125}I-BSA was prepared according to Ref. [32] and then treated as described above for native BSA.

ATP-dependent proteolysis [22,24] was measured as previously described and modified [11] for the measurement of non-ATP-depleted extracts. In this modified test the activity is measured in the presence and absence of 10 mM EDTA. EDTA disrupts the ATP/Mg^{2+} complex by chelating Mg^{2+}. We therefore distinguish ATP/Mg^{2+}-dependent (absence of EDTA) from ATP/ Mg^{2+}-independent activity (= presence of EDTA). The ratio of dependent to independent activity is termed 'ATP-ratio'. In the heart model a possible Mg^{2+}-dependent activity was previ-

Table 2
ATP-dependent proteolytic activity in crude extracts of rabbit tissues [b]

Tissue	Protein	Net ATP-dependent proteolysis			
		Activity		ATP-activity ratio	Approx. tissue contents
	mg/ml	arb. U/ml	arb. U/mg	$\frac{+\text{ATP/Mg}^{2+}}{-\text{ATP/Mg}^{2+}}$	arb. U/g ww
Reticulocytes [a]	34.1	17.4 ± 0.7	0.51	> 6.0	87
Uterine smooth muscle	12.1	10.4 ± 0.6	0.86	4.1	26
Red skeletal muscle	12.7	12.7 ± 0.5	1.0	3.4	26
Erythrocytes [a]	29.5	3.8 ± 0.4	0.13	> 6.0	19
Kidney	19.7	6.5 ± 0.6	0.33	3.3	17
Cardiac muscle	10.3	9.1 ± 0.2	0.88	6.6	17
Brain (cerebrum)	10.4	7.9 ± 0.3	0.76	5.5	14
White skeletal muscle	17.4	7.7 ± 0.5	0.44	4.4	14
Liver	31.0	5.9 ± 1.1	0.19	4.0	12
Spleen	8.9	3.0 ± 0.3	0.34	3.0	10
Eye lens	106.0	7.4 ± 0.1	0.07	2.2	10
Lung	27.5	3.9 ± 1.1	0.14	3.6	8
Testis	10.9	2.5 ± 0.3	0.23	2.0	7

[a] Arb. U/g packed cells ($3000 \times g$) not per g wet weight (ww).

[b] Proteolytic activity was measured with ^{125}I-BSA. Incubation mixtures in a final volume of 200 μl contained: 50 mM Tris/HCl, 1.25 mM DTE, 5 mM $MgCl_2$, 5 mM ATP and 0.01 mg/ml ^{125}I-BSA (10^7 cpm/mg). The final protein concentration of the extract in the incubation mixture was one half of the protein concentration given in the table. Controls were run without ATP/Mg^{2+}. Incubation was stopped after 60 min. The activity (±SE) was calculated from the linear portions of activity progress curves (see Fig. 2) by linear regression analysis ($n = 5$ time points including origin). A similar percentual error applies to the derived quantities (i.e. specific activity and Organ contents). The ATP-independent activity has been subtracted so that the net ATP-dependent activity is given in the table. In those cases where the ATP-independent activity was close to zero the values of the ATP-ratio are given as '> 6.0'. For further details and definition of arb. U see Section 3, the text and [11].

Table 3
Influence of ubiquitin on the ATP-dependent proteolytic activity in APF II of rabbit tissues [b]

Tissue	Protein mg/ml	Net ATP-dependent proteolytic activity		ATP activity ratio $\frac{+ATP/Mg^{2+}}{-ATP/Mg^{2+}}$	Ubiquitin activity ratio $\frac{+Ubi}{-Ubi}$
		arb. U/ml	arb. U/mg		
Reticulocytes [a]	10.0	23.0 ± 0.6	2.3	6.2	5.5
Uterine smooth muscle	7.1	2.8 ± 0.6	0.4	2.2	0.9
Red skeletal muscle	3.3	6.6 ± 0.5	2.0	3.1	0.9
Erythrocytes [a]	1.8	7.0 ± 0.5	3.9	3.0	1.0
Kidney	5.3	3.2 ± 0.3	0.6	2.9	0.9
Cardiac muscle	3.9	5.9 ± 0.4	1.5	4.0	1.2
Brain (cerebrum)	4.2	4.6 ± 0.4	1.1	2.4	0.8
White skeletal muscle	2.4	4.6 ± 1.1	1.9	2.0	1.1
Liver	7.4	7.4 ± 1.6	1.0	3.5	0.8
Spleen	2.9	3.5 ± 0.3	1.2	2.5	1.0
Eye lens	29.4	2.9 ± 0.1	0.1	1.6	1.1
Lung	2.6	0.8 ± 0.1	0.3	1.5	1.2
Testis	5.4	4.4 ± 0.4	0.8	4.5	1.0

[a] Arb. U/g packed cells ($3000 \times g$) not per g wet weight (ww).
[b] APF II was prepared from the respective corresponding extract of each tissue as depicted in Table 2. Proteolytic activity was measured with ^{125}I-BSA. Incubation mixtures in a final volume of 200 μl contained: 50 mM Tris/HCl, 1.25 mM DTE, 5 mM $MgCl_2$, 5 mM ATP and 0.01 mg/ml ^{125}I-BSA (10^7 cpm/mg). Ubiquitin concentration was 0.1 mg/ml. Controls were run without ATP/Mg^{2+}. The concentration of the enzyme solution was one half of the given protein concentration. For further details and definition of arb. U see Section 3, Fig. 3, legend to Table 2 and Ref. [11].

ously excluded with non-hydrolyzable ATP-analogs [11]. The incubation mixture (total vol.: 0.2 ml) for ATP/Mg^{2+}-dependent activity contained as final concentrations: 50 mM Tris/HCl, pH 8.0, 1 mM DTE, 5 mM ATP, 5 mM $MgCl_2$, 10 μg/ml ^{125}I-BSA and 100 μl sample of tissue extracts. Mixtures with buffer instead of sample were incubated as controls. For ATP/Mg^{2+}-independent activity 10 mM EDTA

Table 4
Proteolysis of reductively methylated ^{125}I-BSA by APF II of several tissues [a]

Tissue	Substrate	Proteolytic act.		Net ATP-dependent proteolysis	Substrate act. ratio
		+ ATP/Mg^{2+} arb. U/mg	− ATP/Mg^{2+} arb. U/mg	arb. U/mg	BSA/BSA_{met}
Reticulocyte	BSA	2.86	0.11	2.75	39.3
	BSA_{met}.	0.22	0.15	0.07	
Cardiac muscle	BSA	1.50	0.47	1.03	1.0
	BSA_{met}.	1.60	0.58	1.02	
Liver	BSA	0.54	0.26	0.28	0.9
	BSA_{met}.	0.53	0.21	0.32	
Eye lens	BSA	0.33	0.20	0.13	1.1
	BSA_{met}.	0.36	0.24	0.12	

[a] For preparations of APF II and reductively methylated ^{125}I-BSA see Section 3 and [11]. Proteolytic activity was measured with ^{125}I-BSA. Incubation mixtures in a final volume of 200 μl contained: 50 mM Tris/HCl, 1.25 mM DTE, 5 mM $MgCl_2$, 5 mM ATP, 0.01 mg/ml ^{125}I-BSA (10^7 cpm/mg) and 0.1 mg/ml ubiquitin. The concentration of reticulocyte APF II was 5.0 mg/ml, cardiac muscle APF II 2.5 mg/ml, eye lens APF II 14.7 mg/ml and liver APF II 3.7 mg/ml. Activity was calculated after 60 min of incubation. For further details and definition of arb. U see Section 3 and [11].

was added to the above mixture. When necessary 0.1 mg/ml (final concentration) ubiquitin was added to this standard assay. After incubation at 37°C for a given time (5–120 min) 0.25 ml 10% w/v TCA and 0.05 ml unlabeled BSA (30 mg/ml) were added to the mixtures and kept for 20 min at 0°C. After centrifugation for 2 min in an Eppendorf centrifuge 5415 (Eppendorf, Hamburg) at $16\,000 \times g$ the TCA-soluble radioactivity in the supernatant was measured in a gamma counter (Packard). Activity is expressed in arb. U: 1 arbitrary unit corresponds to % proteolysis/h in the standard incubation mixture of 200 μl [11]. The % proteolysis corresponds to the TCA-soluble radioactivity divided by the TCA-precipitable radioactivity in the incubation mixture (= 100%) at the beginning of the experiment (i.e. at time = 0). The activity of 1 arb. unit/ml (or mg protein) corresponds to the activity per ml or mg of sample solution [11]. The activity can be calculated according to the following equation:

$$\frac{\text{arb. unit}}{\text{ml}} = \frac{\%\text{proteolysis} \times \text{assay vol. (ml)}}{\text{standard assay vol. (0.2 ml)} \times \text{sample vol. (ml)} \times \text{time (h)}} \quad (2)$$

In progress curves the proteolysis of ^{125}I-BSA is expressed in % under the standard conditions described. For activity calculations from the progress curves (7–9 data points) the initial 4 data (assay) points plus the origin were analyzed by linear regression. The derived increments ± standard error (SE) were employed for the respective calculations.

3.2.1.2. Ubiquityl-calmodulin synthetase. UCaM-synthetase activity was measured with the Fluphenazine-Sepharose test, i.e. FP-test [2,33]. The incubation mixture (50–100 μl) contained 50 mM Tris/HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 10 mM phosphocreatine, 0.1 mg/ml creatine kinase, 500 μg/ml calmodulin, 0.05 mg/ml ^{125}I-CT-ubiquitin (specific radioactivity 600–7000 cpm/pmol), 0.9 mg/ml APF II or a protein fraction as indicated at pH 8.0. The mixtures with calcium ($+Ca^{2+}$) contained 1.1 mM $CaCl_2$ and 1 mM EGTA. The mixtures without calcium ($-Ca^{2+}$) only contained 1 mM EGTA. After incubation at 37° for 60 min (other times are indicated in the legend) the mixture was heated to 96°C for 5 min. The samples were placed on ice for 5 min, the denatured protein was spun down on an Eppendorf centrifuge (see above) and the supernatant adsorbed to FP-Sepharose and eluted for analysis as described previously [2,33]. The amount of FP-Sepharose employed depended on the amount of calmodulin in the mixture and was held at the ratio of 100 μl packed FP-Sepharose per 100 μg of calmodulin. Enzymatic activity of uCaM-synthetase corresponds to the transfer of one mol ubiquitin to calmodulin per second and is expressed in katal. The progress curves were analyzed as described above for the proteolytic activity.

3.2.1.3. Deconjugation of calmodulin. The incubation mixture contained in a final volume of 25–100 μl: 50 mM Tris/HCl, 1 mM DTE, pH 8.0 and the indicated amounts of enzyme solution. After incubation at 37°C for 30–60 min the mixtures were analyzed by 15% SDS-PAGE and autoradiography (see below). Further details are given in the legend.

3.2.1.4. Proteolysis of calmodulin. In these experiments the Bolton-Hunter method of ^{125}I-labeling of calmodulin was employed because its Ca^{2+}-dependent ubiquitylation properties are fully retained [27]. The proteolysis of ^{125}I-BH-calmodulin was measured similarly as described above for the assay of ATP-dependent proteolysis with ^{125}I-BSA except that the ^{125}I-BH-calmodulin concentration was increased 10-fold to 100 μg/ml (ca. $K_{0.5}$ for uCaM synthetase). The incubation mixture of 200 μl contained 50 mM Tris/HCl, pH 8.0, 1 mM DTE, 5 mM ATP, 5 mM $MgCl_2$, 1 mM EGTA, 16 mg/ml reticulocyte lysate and as substrate either 10 μg/ml ^{125}I-BSA (control), 100 μg/ml ^{125}I-BH-calmodulin (^{125}I-CaM; specific radioactivity $2.1 \cdot 10^4$ cpm/μg) or methylated ^{125}I-BH-calmodulin (^{125}I-CaM-m; specific radioactivity $1.9 \cdot 10^4$ cpm/μg) respectively. Alternatively the mixtures contained additionally 1.1 mM $CaCl_2$, (= $+Ca^{2+}$) or 10 mM EDTA (= $-ATP/Mg^{2+}$). The mixtures were incubated at 37°C for the indicated times, precipitated with TCA and the soluble radioactivity was measured as described above.

3.2.2. Other procedures

3.2.2.1. Protein. Protein was determined after TCA precipitation (5%), washing and resolubilization of the pellet in 0.1 M NaOH, 1% SDS according to the

method of [34] on an AutoAnalyzer (Technicon) employing BSA as standard.

3.2.2.2. SDS-PAGE autoradiography. Unless otherwise stated polyacrylamide gel electrophoresis in the presence of SDS was performed on 15% gels according to [35] and the sample buffer for the Laemmli system [35] additionally contained 10 mM EGTA [2].The molecular weight standards for SDS-PAGE are given under Materials (see above). ^{125}I-ubiquityl-calmodulin was determined by quantitative autoradiography [23]. The obtained autoradiograms (maximum absorbance 1.8 *A*) were densitometered at 659 nm (full scale: 3.0 *A*, speed 15 mm/min) on a laser scanner (Ultroscan 2202, Pharmacia, Freiburg). The data was directly transferred to a Shimadzu Integrator C-R3A Chromatopac (Shimadzu, Düsseldorf) for conversion (settings: speed 30 mm/min, slope 10 μV/min, drift 0 μV/min, attenuation 9, width 20 s) to peak area in μV^2. For conversion of the peak area to pmol ubiquityl calmodulin conjugate the system was calibrated as described in ref. [1]. For autoradiography the undried gels (without prior staining or destaining) were placed between two layers of plastic foil in the presence of enhancer foils (Cronex Lightning Plus, Dupont) and were exposed to X-ray films (Fuji RX, NIF) at −80°C for 24–96 h as required and developed as described [1,2,11].

3.2.3. Nomenclature

The nomenclature employed follows the suggestions made in [4].

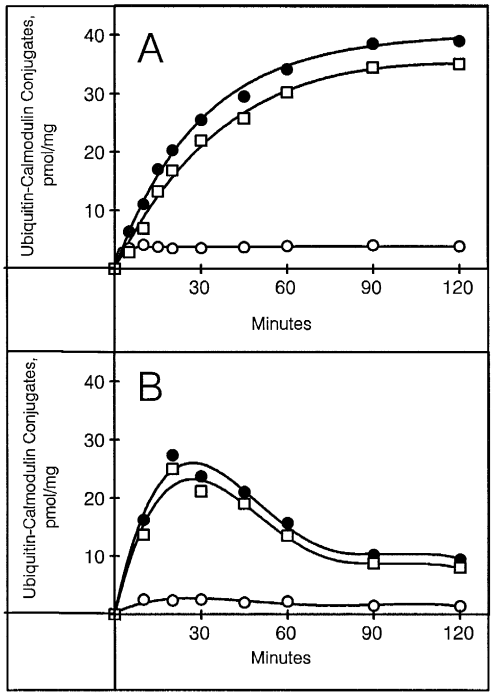

Fig. 1. Progress curves of the ubiquitylation of calmodulin in reticulocyte lysate (A) and spleen crude extract (B). The incorporation of ubiquitin into calmodulin (CaM) was measured with the FP-test (see Section 3 and Refs. [2,33]). The incubation mixture contained in a final volume of 100 μl: 50 mM Tris/HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 10 mM phosphocreatine, 0.1 mM $CaCl_2$, 0.1 mg/ml creatine kinase, 0.5 mg/ml calmodulin, 6.7 mg/ml reticulocyte lysate or 6.7 mg/ml spleen crude extract, respectively, and 0.05 mg/ml ubiquitin ($6 \cdot 10^7$ cpm/mg). Controls were run without calmodulin. (○) control (only endogenous calmodulin), (●) + exogenous calmodulin, (□) net conjugation of exogenous calmodulin.

4. Results

4.1. Ubiquityl-calmodulin synthetase in rabbit tissues

For determination of catalytic rates the kinetics of the conjugation of calmodulin with ubiquitin were measured over a time of 120 min for each organ tested. Since not all of these progress curves can be presented, representative data is shown for two tissues. The activity of uCaM-synthetase in reticulocyte lysate and spleen crude extract are shown in Fig. 1. In lysate (Fig. 1A) the conjugation of ubiquitin to calmodulin increases linearly for about 30 min and then reaches a plateau level. The activity of uCaM synthetase can be calculated from the linear portion of the curve. In the spleen crude extract (Fig. 1B) the amount of calmodulin ubiquitylated also initially increases proportionally to the time approaching a plateau but then runs through a maximum at ca. 25 min to decrease from 30 pmol/mg to 10 pmol/mg at 120 min. These latter results indicate that the amount of conjugate formed in the plateau region or maximum corresponds not to an equilibrium but to a steady state level due to an unknown counteracting reaction. In 50% of the tissue extracts tested such maximum time curves (see Fig. 1B) could be detected (also see Table 1).

The uCaM synthetase activities in various rabbit tissue extracts calculated from the initial increments

(first 20–30 min) of progress curves are shown in Table 1 in the order of the tissue contents. The highest specific activities of uCaM synthetase (14–21 fkat/mg) are found in reticulocytes and spleen. The specific activity of the enzyme in liver is over 6-fold and in eye lens over 45-fold lower respectively. The highest tissue contents (per g wet weight) are again found in reticulocytes (2339 fkat/g ww) and spleen 933 fkat/g ww. A low content was measured in striated muscles and the eye lens. We then asked the question if the tissue content of uCaM Synthetase correlated with the calmodulin contents in mammalian tissues [36]. Apart from reticulocytes, where the exact tissue content of calmodulin has only estimated, the spleen with a low calmodulin content of ca. 80 μg/g has a high synthetase activity yielding a synthetase/calmodulin activity ratio of 11.7. In contrast liver with the same tissue content of calmodulin only has an activity ratio of 1.6. Tissues with the highest contents in calmodulin such as brain or testis have a low synthetase/calmodulin activity ratio of 0.4–0.5 similar to white skeletal muscle which however has one of the lowest calmodulin contents (see Table 1). Thus there appears to be no correlation between uCaM synthetase activity content and the calmodulin content of these tissues.

4.2. ATP-dependent proteolysis in rabbit tissues

Since ATP-ubiquitin-dependent proteolysis could be the likely cause for decreasing ubiquitin-calmodulin conjugates and thus a steady-state of conjugates, the same tissue extracts (see Table 1) were assayed for ATP-dependent proteolytic activity with the classical substrate ^{125}I-BSA and some representative progress curves are shown. The proteolytic activity in reticulocyte lysate and uterine smooth muscle crude extracts in the presence and absence of ATP is depicted in Fig. 2A and B, respectively. The proteolytic activity in reticulocyte lysate displays a distinct lag phase. In addition proteolysis in the absence of ATP is often close to zero with freshly prepared substrate (Fig. 2A) leading to very high apparent ATP-ratios. In smooth muscle extracts (Fig. 2B) a linear proteolysis phase is followed by a plateau phase and in addition a significant amount of ATP-independent proteolytic activity can be measured. The ATP-dependent activity is of the same magnitude as that in reticulocyte lysate (Fig. 2A). Typically the progress curves remain linear for 30–60 min allowing for the calculation of the activities in Table 2. As can be seen the ATP-ratio observed lies between 2.0 and >6.0 clearly demonstrating that ATP-dependent proteolytic activity can be detected in all of these tissues. The highest tissue content of ATP-dependent proteolytic activity is found in reticulocytes (87 arb. U/g ww). For other tissues the content in ATP-dependent proteolysis varies between 7–26 arb. U/g ww. The ca. 4-fold decrease of the total activity in erythrocytes versus reticulocytes is probably due to the disappearance of the ATP-ubiquitin-dependent proteolysis system [37].

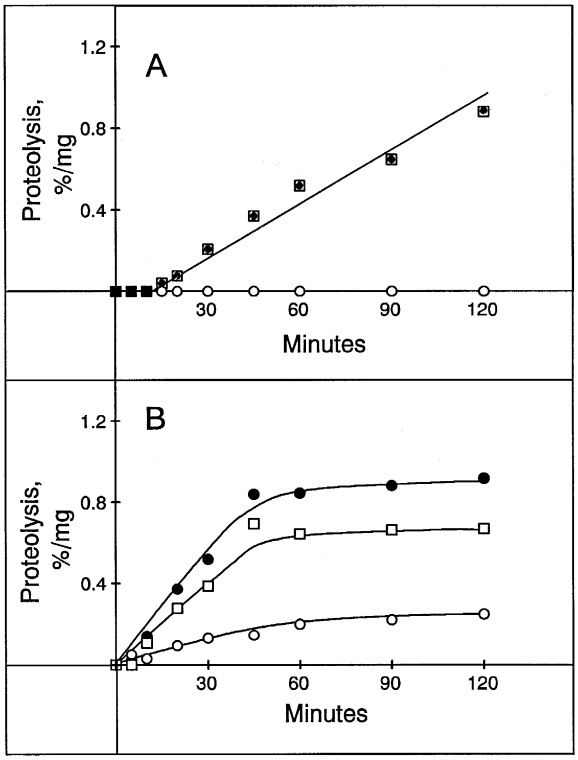

Fig. 2. Progress curves of ATP-dependent proteolysis of ^{125}I-BSA in reticulocyte lysate (A) and uterine smooth muscle crude extract (B). Proteolytic activity was measured with ^{125}I-BSA. Incubation mixtures in a final volume of 200 μl contained: 50 mM Tris/HCl, 1.25 mM DTE, 5 mM $MgCl_2$, 5 mM ATP, 0.01 mg/ml ^{125}I-BSA (10^7 cpm/mg) and either 17.1 mg/ml reticulocyte lysate or 6.1 mg/ml uterine smooth muscle crude extract respectively. Controls were run without ATP/Mg^{2+}. For further details see Section 3 and [11]. (○)-ATP, (●) +ATP, (□) net ATP-dependent activity.

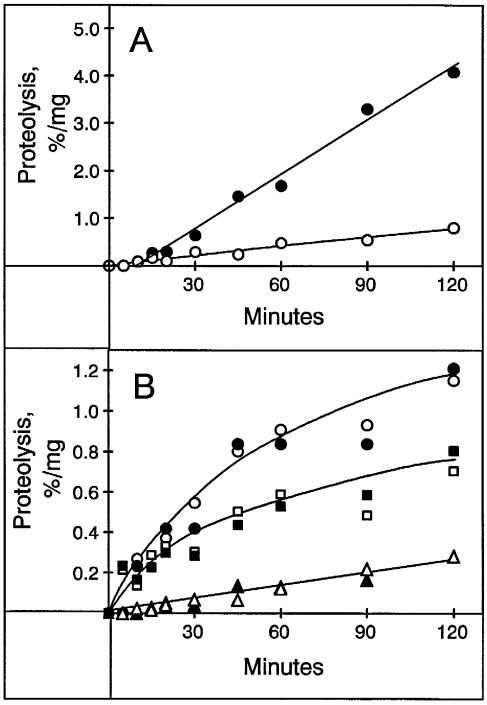

Fig. 3. Influence of ubiquitin on the ATP-dependent proteolytic activity of reticulocyte APF II (A), cardiac muscle APF II, kidney APF II and eye lens APF II (B). Net ATP-dependent proteolytic activity was measured with ^{125}I-BSA. Incubation mixtures in a final volume of 200 μl contained: 50 mM Tris/HCl, 1.25 mM DTE, 5 mM $MgCl_2$, 5 mM ATP, 0.01 mg/ml ^{125}I-BSA (10^7 cpm/mg) and either 5.0 mg/ml reticulocyte APF II, 2.2 mg/ml cardiac muscle APF II, 2.7 mg/ml kidney APF II or 14.7 mg/ml eye lens APF II respectively. When indicated 0.1 mg/ml ubiquitin was added. For further details see Section 3 and Ref. [11]. A. reticulocyte APF II: (○) control (−ubiquitin), (●) +ubiquitin. B. cardiac muscle APF II: (○) control (−ubiquitin), (●) +ubiquitin, eye lens APF II: (△) control (−ubiquitin), (▲) +ubiquitin, kidney APF II: (□) control (−ubiquitin), (■) +ubiquitin.

In order to establish whether this proteolytic activity is ubiquitin-dependent, endogenous ubiquitin in the extracts was removed by DEAE chromatography (see methods). As shown in Fig. 3A and Table 3 proteolysis in reticulocyte APF II is stimulated 5–6-fold by ubiquitin. A slight lag period is observed in the first min. In contrast no activation by ubiquitin (ubiquitin activation ratio $\sim$ 0.8–1.2) could be found in cardiac muscle, kidney, eye lens (Fig. 3B) or in any of the other tissue extracts tested in Table 3. On the other hand the ATP ratios especially between 2.5 and 4.5 strongly indicate the presence of another ubiquitin-independent ATP-dependent proteolytic activity.

A possibility which had to be excluded was that some residual endogenous ubiquitin had remained in the enriched extracts leading to apparent independence from exogenous ubiquitin. Therefore methylated BSA, which cannot be ubiquitylated, was tested exemplary in the APF II of three tissues (Table 4). Only in reticulocyte APF II employed as control is the ATP dependent proteolytic activity drastically reduced (ca. 40-fold) when the free -amino groups in the substrate BSA have been methylated (for degree of methylation see [27]). In contrast the energy dependent proteolytic activity in the DEAE enriched extracts of cardiac muscle, liver and eye lens is not influenced by BSA methylation at all thus excluding a ubiquitin conjugating mechanism.

4.3. Dynamics of ubiquitin-calmodulin conjugates in cell-free extracts

Since it could be that ^{125}I-BSA behaves differently from calmodulin as a substrate in proteolytic assays, calmodulin was tested directly to see if the decline in activity seen in the maximum curve (Fig. 1B) could be a result of the ubiquitin pathway of proteolysis. ^{125}I-BH-calmodulin was chosen, since labeling by the Bolton-Hunter procedure does not alter the substrate properties of calmodulin for uCaM-synthetase [27]. Reticulocyte APF II was chosen as a representative tissue fraction since it contained a high uCaM-synthetase activity (Table 1) and was the only fraction to exhibit ATP-ubiquitin-dependent proteolytic activity (Table 3). As is demonstrated in Fig. 4 no influence of ubiquitin on the proteolysis of ^{125}I-BH-calmodulin could be detected in the presence or absence of ATP whether high or low Ca^{2+} concentrations were chosen (Fig. 4A,B). In the absence of Ca^{2+} a significant ATP-dependent ubiquitin-independent proteolysis of calmodulin is found (Fig. 4B) which is of a similar activity level as the ubiquitin-dependent proteolysis of ^{125}I-BSA (Fig. 4C) and which may indicate the existence of a similar ubiquitin-independent protease in reticulocytes as that found in the other tissues (Table 3).

Since a protein component necessary for the degradation of calmodulin could have been removed by the ion-exchange procedure employed for the preparation

of reticulocyte APFII, the proteolysis of calmodulin was examined directly in lysate. As shown in Table 5 the proteolysis of ^{125}I-BH-calmodulin in the presence of Ca^{2+} i.e. under conditions of calmodulin conjugation, is ATP-independent. The gross proteolytic rate under these conditions is ca. 8-fold lower than the proteolysis of the control substrate ^{125}I-BSA, although on a weight basis the concentration ^{125}I-BH-calmodulin in the assay was 10-fold higher than that of ^{125}I-BSA. As in the enriched APF II (Fig. 4) ATP-dependent proteolysis of ^{125}I-BH-calmodulin is clearly ca. 2.5–3.0-fold higher in the absence of Ca^{2+} (i.e. 3.1%/(ml × h) than in the presence of Ca^{2+} i.e. under conditions where ubiquitylation of calmodulin does not take place. From this value it can be calculated that 0.19% of the calmodulin is degraded per hour and per mg lysate protein with 50% of the calmodulin being degraded per mg lysate protein in 250 h (i.e. apparent half-life). Finally as shown in Table 5 methylation of calmodulin, which prohibits ubiquitylation, shows no influence on the proteolysis of BH-calmodulin in reticulocyte lysate in the presence of ATP thereby yielding a final argument for excluding the ubiquitin pathway as the mode of degradation also in the cell-free extract of reticulocytes (lysate).

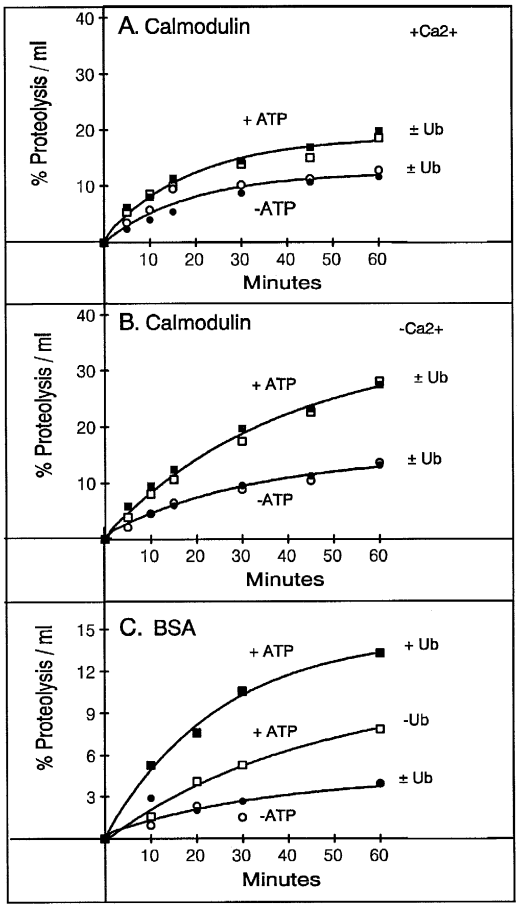

Fig. 4. Influence of ubiquitin on the ATP-dependent proteolysis of ^{125}I-BH-Calmodulin and ^{125}I-BSA by reticulocyte APF II. Incubation mixtures in a final volume of 200 μl contained: 50 mM Tris/HCl, 1.25 mM DTE, 5 mM $MgCl_2$, 5 mM ATP, 1 mM EGTA, 1.7 mg/ml reticulocyte APF II and as substrate either 100 μg/ml ^{125}I-BH-calmodulin ($2.1 \cdot 10^4$ cpm/μg) in A and B or 10 μg/ml ^{125}I-BSA ($1.8 \cdot 10^4$ cpm/μg) in C. Alternatively the mixtures contained in addition 1.1 mM $CaCl_2$ (= $+Ca^{2+}$), 10 mM EDTA (= $-ATP/Mg^{2+}$) or 0.1 mg/ml ubiquitin. The mixtures were incubated at 37°C. At the indicated times aliquots of 20 μl were taken and precipitated with TCA. TCA soluble peptides in the supernatant were counted as described in Methods. A. Proteolysis of ^{125}I-BH-calmodulin + Ca^{2+}. B. Proteolysis of ^{125}I-BH-calmodulin-Ca^{2+} (+EGTA). C. Proteolysis of ^{125}I-BSA, −ATP: (○) −ubiquitin, (●)+ubiquitin; +ATP: (□) −ubiquitin, (■) +ubiquitin.

Since the decline in calmodulin conjugate level as seen in spleen extract (Fig. 1B) was much more rapid than any of the proteolytic rates observed above indicated, the mechanism underlying the maximum curves was examined in more detail in liver extract, which showed the same behavior as spleen but could be obtained in higher amounts. In Fig. 5 a typical maximum curve in liver crude extract is shown. A maximum is reached after ca. 20 min and then the curve decreases with a half-life of ca. 55 min. Addition of 0.5 mg/ml ubiquitin$_t$, (which cannot be conjugated to proteins), surprisingly increases the amount of conjugate formed after 20 min by nearly 50%. In addition the decline in conjugates between 60–120 min is significantly reduced. The insert in Fig. 5 demonstrates that the radioactivity changes measured truly reflect the changes in conjugate levels present in the incubation mixture.

Fig. 6 shows the influence of various parameters on conjugate development and levels in liver crude extract. The maximum steady state level and following decline of conjugates is dependent on dilution (Fig. 6A) indicating a strong dependence on the concentration of a factor influencing enzyme activity. A 2-fold dilution practically abolishes the decay of conjugates (Fig. 6A). On the other hand addition of 3.0 mg/ml ubiquitin$_t$ to the incubation mixture (Fig.

6B) not only abolishes the decay but strongly enhances the net synthesis of ubiquityl-calmodulin which increases to a new higher steady state. Since ubiquitin$_t$ cannot be conjugated to proteins [24] and evidently does not inhibit uCaM synthetase its effect must be to inhibit the opposing reaction(s) which decrease the concentration of conjugates. Therefore, in a third experiment (Fig. 6C), performed at a higher protein concentration to enhance the decay rate, EDTA was added to chelate Mg^{++}. EDTA which inhibits ubiquitin conjugation and all ATP-dependent proteolytic activity strongly increases the net disappearance of conjugates ($t_{1/2} \sim 30$ min) versus the control (no addition, $t_{1/2} \sim 45$ min). If the calmodulin conjugates were being degraded by an ATP-dependent-protease, then the addition of EDTA should have led to a stabilization of the conjugates at a plateau level. However this is not the case in agreement with the previous experiments above. If ubiquitin$_t$ is added together with EDTA a plateau, stable for 60 min, is obtained (Fig. 6C) due to a simultaneous inhibition of the synthesis and decay of conjugates. EGTA which selectively inhibits the Ca^{2+}-dependent conjugation of calmodulin leads to a somewhat faster decay than EDTA alone ($t_{1/2} \sim 15–20$ min).

These results indicated that a strong activity probably unrelated to the ubiquitin proteolytic pathway, which was too high to be of other proteolytic origin, was counteracting the synthesis of ubiquityl-calmodulin conjugates. Since liver was a tissue, which in our hands did not contain the ATP-ubiquitin-dependent proteolytic pathway in significant amounts,

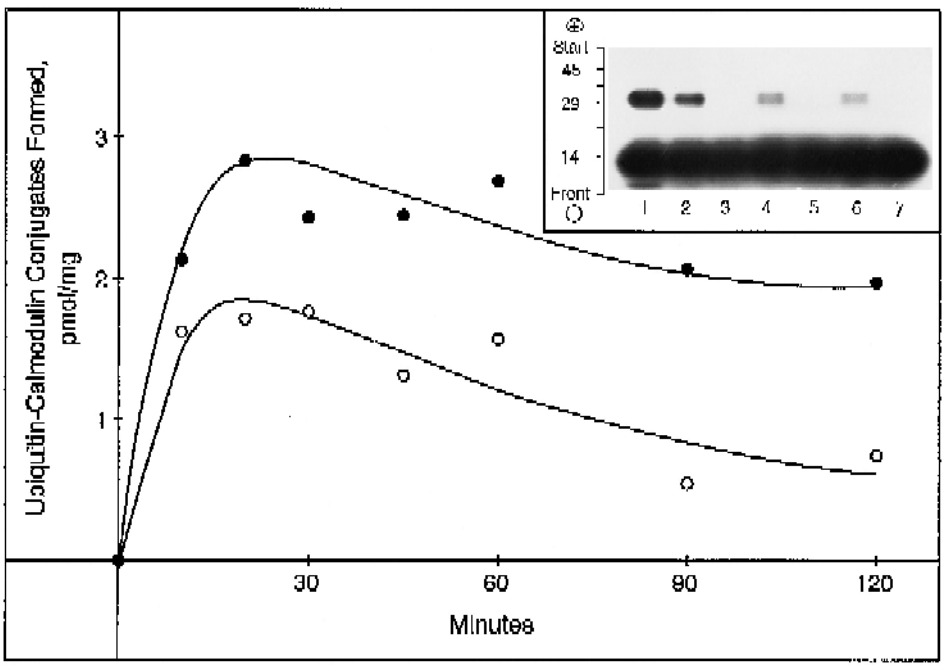

Fig. 5. Progress curves of the ubiquitylation of calmodulin in a liver crude extract. The incorporation of ubiquitin into calmodulin (CaM) was measured with the FP-test (see Section 3 and Refs. [2,33]). The incubation mixture contained in a final volume of 100 μl: 50 mM Tris/HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 10 mM phosphocreatine, 0.1 mM $CaCl_2$, 0.1 mg/ml creatine kinase, 0.5 mg/ml non-labeled ^{125}I-BH-calmodulin, 12.0 mg/ml liver crude extract and 0.05 mg/ml ^{125}I-ubiquitin ($0.3 \cdot 10^8$ cpm/mg). As indicated incubation mixtures contained in addition 0.5 mg/ml ubiquitin$_t$. The efficiency of the FP-test with ^{125}I-BH-calmodulin is ca. 50% less than with ^{125}I-CT-ubiquitin, leading to ca. 2–3-fold lower enzyme actitivities. Controls were run without ATP/Mg^{2+}. Inset: Autoradiogram of the incubation mixture of liver extract at various times for detection of ^{125}I-labeled ubiquitin-calmodulin conjugates. Only the monoconjugate is visualized (see Ref. [23]). Lane 1: reticulocyte APF II (control); lane 2: 20 min +ATP; lane 3: 20 min −ATP; lane 4: 90 min +ATP; lane 5: 90 min −ATP; lane 6: 120 min +ATP; lane 7: 120 min −ATP (○) control (50 μg ^{125}I-ubiquitin/ml), (●) +ubiquitin$_t$ (500 μg/ml).

Table 5
Proteolysis of non-methlyted and methylated ^{125}I-BH-calmodulin by reticulocyte lysate [a]

Substrate	Proteolysis %/(ml×h)			
	+ATP/Mg^{2+}		−ATP/Mg^{2+}	
^{125}	+Ca^{2+}	−Ca^{2+}	+Ca^{2+}	−Ca^{2+}
I-BSA Control		8.8±1.1		2.4±1.0
^{125}I-BH-CaM	1.0±0.3	3.1±0.2	1.1±0.2	1.6±0.3
^{125}I-BH-CaM-m	1.1±0.3	2.5±0.2	0.3±0.3	0.6±0.2

[a] The incubation mixture of 200 μl contained 50 mM Tris/HCl, pH 8.0, 1 mM DTE, 5 mM ATP, 5 mM $MgCl_2$, 1 mM EGTA, 16 mg/ml reticulocyte lysate and as substrate either 10 μg/ml ^{125}I-BSA, 100 μg/ml ^{125}I-BH-calmodulin (^{125}I-BH-CaM; specific radioactivity 2.1×10^4 cpm/μg) or methylated ^{125}I-BH-calmodulin (^{125}I-CaM-m; specific radioactivity 1.9×10^4 cpm/μg) respectively. Alternatively the mixtures contained additionaly 1.1 mM $CaCl_2$, (=+Ca^{2+}) or 10 mM EDTA (=−ATP/Mg^{2+}). The mixtures were incubated at 37°C for 60 min during which 4 samples were taken at different time points and precipitated with TCA and the TCA-soluble peptides in the supernatant counted as described in Section 3.

the above experiments were repeated with a somewhat different objective in reticulocytes, where the ubiquitin pathway could compete with the novel activity. In Fig. 7a isolated ^{125}I-BH-calmodulin labeled ubiquitin conjugates (uCaM, u_2CaM, u_3CaM) were incubated with reticulocyte lysate. Within minutes after the addition of lysate a deconjugation of ubiquitin-calmodulin conjugates which is complete in 40 min leads to the generation of a strong band of intact labeled ^{125}I-BH-calmodulin (Fig. 7A). It should be noted this band is time-stable for 180 min and that no radioactive bands below the native molecular mass of ^{125}I-BH-calmodulin indicative of other proteolytic fragmentation can be detected. We conclude that this conjugate decay can be attributed to a ubiquityl-

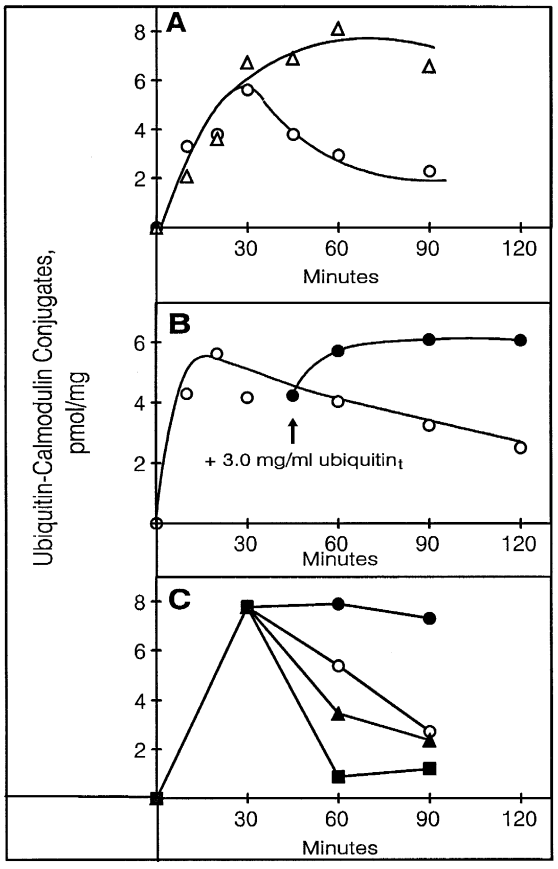

Fig. 6. Influence of dilution, ubiquitin$_t$ and chelators on the steady state level of ubiquityl-calmodulin in liver crude extract. A. Dilution. The incorporation of ubiquitin into calmodulin (CaM) was measured with the FP-test (see Section 3 and Refs. [2,33]). The incubation mixture contained in a final volume of 50 μl: 50 mM Tris/HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 10 mM phosphocreatine, 0.1 mM $CaCl_2$, 0.1 mg/ml creatine kinase, 0.5 mg/ml calmodulin, 12 mg/ml liver crude extract and 0.05 mg/ml ^{125}I-ubiquitin ($0.36\cdot10^8$ cpm/mg). In a second mixture the crude liver extract was diluted 2-fold. Controls were run without ATP/Mg^{2+}. B. Ubiquitin$_t$. The incorporation of ubiquitin into calmodulin (CaM) was measured with the FP-test (see Section 3 and Refs. [2,33]). The incubation mixture contained in a final volume of 100 μl: 50 mM Tris/HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 10 mM phosphocreatine, 0.1 mM $CaCl_2$, 0.1 mg/ml creatine kinase, 0.5 mg/ml calmodulin, 8.8 mg/ml liver crude extract and 0.05 mg/ml ^{125}I-ubiquitin ($0.36\cdot10^8$ cpm/mg). At the indicated times ubiquitin$_t$ in a final concentration of 3.0 mg/ml was added. Controls were run without ATP/Mg^{2+}. C. Chelators. The incorporation of ubiquitin into calmodulin (CaM) was measured with the FP-test (see Section 3 and Refs. [2,33]). The incubation mixture contained in a final volume of 100 μl: 50 mM Tris/HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 10 mM phosphocreatine, 0.1 mM $CaCl_2$, 0.1 mg/ml creatine kinase, 0.5 mg/ml calmodulin, 12.0 mg/ml liver crude extract (same extract as in Fig. 5) and 0.05 mg/ml ^{125}I-ubiquitin ($0.3\cdot10^8$ cpm/mg). After 30 min EGTA, EDTA or EDTA and ubiquitin$_t$ were added as indicated. Controls were run without ATP/Mg^{2+}. A. (○) undiluted control; (△) 2-fold dilution. B. (○) control (50 μg ^{125}I-ubiquitin/ml), (●) +ubiquitin$_t$ (3 mg/ml). C. (○) control (50 μg ^{125}I-ubiquitin/ml), (●) +ubiquitin$_t$ (10 mg/ml) +5 mM EDTA, (▲) +5 mM EDTA, (■) +1 mM EGTA.

calmodulin splitting isopeptidase activity. This isopeptidase activity is sensitive to inhibition by ubiquitin (Fig. 7B) with an estimated concentration of half-maximal inhibition of ca. 0.5 mg/ml ubiquitin in a 10-min assay, thus explaining the experiments in Figs. 6 and 7.

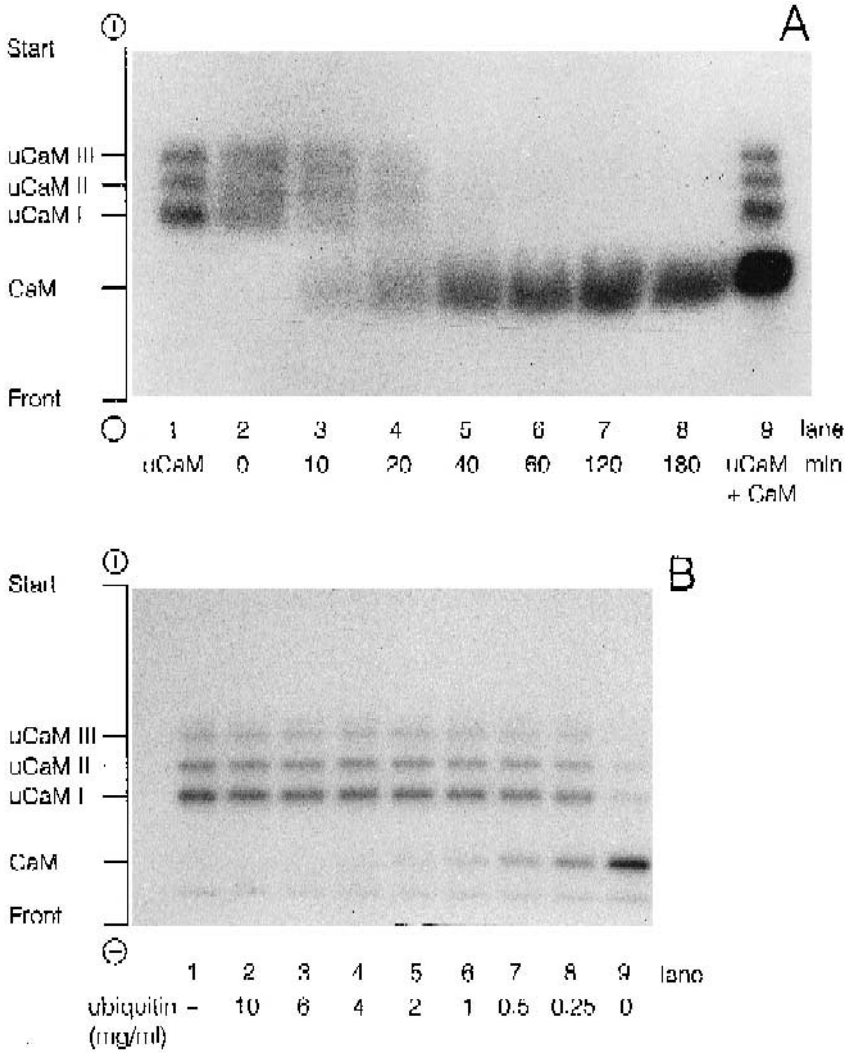

Fig. 7. Evidence for ubiquityl-calmodulin isopeptidase activity in reticulocyte lysate and reticulocyte APF II. A. Incubation mixtures (100 μl) for the hydrolysis of ubiquityl-calmodulin conjugates by uCaM-isopeptidase contained 50 mM Tris, 5 mM ATP, 5 mM $MgCl_2$, 1.25 mM DTE, 0.1 mM $CaCl_2$, 14 μg/ml ^{125}I-BH-calmodulin labeled conjugates (3200 cpm/μg) and 7.1 mg/ml reticulocyte lysate. Control mixtures were run in the presence of 10 mM EDTA (i.e. $-Mg^{2+}$) or 1 mM EGTA (i.e. $-Ca^{2+}$) respectively (not shown as autoradiogram). Incubation was stopped at the indicated times (see graph) by adding 60 μl of Laemmli sample buffer containing 10 mM EGTA and heating to 96°C for 5 min. The reaction mixtures were then analyzed on a 15% SDS-PAGE followed by autoradiography. For further details on the incubation mixture of isopetidase and the isolation of substrate conjugates see Section 3 and the text. Lane 1: 1.4 μg ubiquityl-^{125}I-BH-calmodulin. Lanes 2–8: incubation time 0 to 180 min. Lane 9: 1.4 μg ubiquityl-^{125}I-BH-calmodulin + 1 μg ^{125}I-BH-calmodulin. B. Incubation mixtures (25 μl) contained 50 mM Tris, 1 mM DTE, 0.1 mM $CaCl_2$, 71 μg/ml ^{125}I-BH-calmodulin labeled conjugates (4900 cpm/μg), 0.6 mg/ml reticulocyte APF II and the indicated amounts of ubiquitin. Incubation was stopped after 10 min by adding 60 μl of Laemmli sample buffer containing 10 mM EGTA and heating to 96°C for 5 min. The reaction mixtures were then analyzed on a 12.5% SDS-PAGE followed by autoradiography. Lane 1: no APF II added. Lane 2: 10.0 mg/ml ubiquitin. Lane 3: 6.0 mg/ml ubiquitin. Lane 4: 4.0 mg/ml ubiquitin. Lane 5: 2.0 mg/ml ubiquitin. Lane 6: 1.0 mg/ml ubiquitin. Lane 7: 0.5 mg/ml ubiquitin. Lane 8: 0.25 mg/ml ubiquitin. Lane 9: 0.0 mg/ml ubiquitin.

5. Discussion

It was shown that the ubiquitin-calmodulin ligase system or uCaM-synthetase as it is also called, can be found in the extracts of all 13 rabbit tissues tested (see Table 1). The results suggest the conclusion that all tissues containing calmodulin also contain uCaM-synthetase. This would be in agreement with the general finding that calmodulins from plants, fungi and yeast can be ubiquitylated by uCaM synthetase [27,33] and that this enzyme activity could be identified in yeast [33,38]. UCaM synthetase therefore appears to play an important but as yet not fully understood role in all eukaryote cells. One possible function, namely a negative modulation of the biological activity of calmodulin has been suggested [5] but remains to be substantiated.

Similarly we have detected an ATP-dependent proteolytic activity degrading ^{125}I-BSA in the tissue extracts tested (see Table 2). However, except in the case of reticulocytes, this activity was ubiquitin independent (see Table 3). At present the generally accepted ATP-dependent protease in the cytosol is the ATP-dependent-26-*S*-protease [39]. Together with the ubiquitin-protein ligase system and in some cases with the arginyl-tRNA-protein transferase it constitutes the ATP-ubiquitin-dependent proteolytic pathway [6]. The question arises why no ubiquitin-dependent proteolysis can be detected in any other tissue extract except that from reticulocytes (Table 3). A possible answer is that one of the enzymes involved in the ubiquitin-dependent degradation of ^{125}I-BSA in reticulocytes is only present at a very low level or even possibly missing in the other tissues. Since it has been reported that the arginyl-tRNA-protein transferase is present in all mammalian cells [40] it is improbable that arginylation is limiting. Similarly the 20S multicatalytic endopeptidase complex, the core of the ATP-dependent-26-*S*-protease, is a constituent of all eukaryotic cells [41,42]. It therefore appears unlikely that the ATP-dependent-26-*S*-protease is lacking. Since the proteolysis-connected isopeptidase activity in the ubiquitin system has been shown to be directly associated with the ATP-dependent-26-*S*-protease [43] it is also unlikely that this activity is lacking. Thus the ubiquitin-protein ligase system itself [44] emerges as the possible missing candidate. A reduced or absent activity of this enzyme system in differentiated tissues would be in agreement with the finding that in red cells ubiquitin protein ligase disappears during differentiation from reticulocytes to erythrocytes [37,45]. The findings of this paper are therefore in agreement with the suggestion [4] that all differentiated cell systems i.e. tissues/organs can be compared biochemically to erythrocytes where ubiquitin-protein ligase activity is very low or absent.

The absent ubiquitin dependence of proteolysis found in this paper does not appear due to a proteolytic inactivation of ubiquitin (removal of C-terminal Gly-Gly leading to 'des-Gly-Gly ubiquitin') as reported by [46] for liver extracts, since the proteolysis assays were performed at pH 8.0 [11]. At this pH value the responsible cathepsin (pH optimum = 5.0) is itself inactive and is additionally rapidly and irreversibly inactivated [46]. In addition all extracts were prepared in the presence of leupeptin (see Methods).

Our results with methylated BSA (Table 4) which also exclude the ubiquitin mechanism in the breakdown of ^{125}I-BSA in the tissue extracts, are supported by the microinjection studies of ^{125}I-BSA and ^{125}I-lysozyme into hepatoma tissue culture cells as described by [47]. They found that methylation of the amino groups of ^{125}I-BSA and ^{125}I-lysozyme had no effect on the intracellular degradation rates of these two proteins. They therefore concluded that ^{125}I-BSA and ^{125}I-lysozyme even if microinjected as foreign proteins into hepatoma cells are not degraded by the ATP-ubiquitin-dependent proteolytic pathway [47].

The question therefore arises how the detected ATP-dependent proteolytic activity in the various tissues can be explained. A ubiquitin-independent degradation by the ATP-dependent-26-*S*-protease by a mechanism similar to the antizyme-dependent degradation of ornithine decarboxylase [48] is unlikely. In addition purified ATP-dependent-26-*S*-protease is incapable of degrading ^{125}I-labeled casein [48], ^{125}I-labeled lysozyme [48] and ^{125}I-labeled BSA [39]. Since the purified 20S multicatalytic-endopeptidase-complex ($\sim$ 700 kDa) only catalyzes ATP-independent proteolytic reactions [39,41,49,50] and can in fact even be inhibited by ATP [14,41,51] it is also not a good candidate for the observed ATP-dependence.

Since however in this paper the same substrate and conditions have been used by us as in our first paper on cardiac muscle [11] we feel that the 310–350 kDa

ATP-dependent protease reported by us as 'proteokinain' several years ago [11,14] may be the basis of the observed ATP-dependent degradation of ^{125}I-BSA in the non-methylated as well as in the methylated form. For this proteolytic activity it was also demonstrated with non-hydrolyzable ATP-analogs that activity is only expressed with the hydrolyzable ATP-moiety [11]. Besides our work [11,14] ubiquitin-independent ATP-dependent proteolytic systems have also been described by other groups [9,10,12,13] and it cannot be excluded that a mixture of different proteases of this type exists in tissues. However these systems are not yet clearly defined and a molecular mass of ca. 300 kDa was not reported by the other groups.

Kuehn et al. 1989 [49] reported that part of their ATP-dependent proteolytic activity found in reticulocytes appeared due to a contamination with a cysteine proteinase complexed to α_1-macroglobulin from residual blood proteins. Since the proteolytic activity detected by us in reticulocytes can be specifically stimulated 5–6-fold by ubiquitin (Table 3) a contamination even of our blood material with proteinase-α_1-macroglobulin complexes seems unlikely. In addition the animals used in our study were immediately and completely exsanguinated after sacrificing in order to reduce residual blood in the tissues and thereby to eliminate a possible contamination by cysteine proteinase-α_1-macroglobulin complexes. In this connection we also examined eye lens (Tables 3 and 4) which is neither vascularized nor contains leukocytes [52]. In this tissue where a contamination by cysteine proteinase-α_1-macroglobulin complexes can be excluded we found a low but significant ATP-dependent proteolytic activity.

One of the most intriguing findings of this paper concerns the dynamic state of calmodulin conjugates in the various tissues as demonstrated by so-called maximum curves in 50% of the tissues tested (Fig. 1, Table 1). EDTA (which inactivates the ATP-dependent-26-*S*-protease) decreases the half-life of calmodulin conjugates from $\sim$45 min to $\sim$30 min instead of stabilizing the conjugate level. This therefore makes the ATP-dependent-26-*S*-protease pathway improbable as the major route of conjugate decomposition. This is conclusively supported by the experiment in Table 5. In reticulocyte lysate which contains sufficient endogenous ubiquitin, an increase in the ATP-dependent proteolysis of calmodulin in the presence of Ca^{2+} i.e. under ubiquitylating conditions cannot be detected. In addition methylation of calmodulin does not reduce the proteolysis rate under these conditions. In contrast it is consistently found that proteolysis of calmodulin is higher in the absence of Ca^{2+} i.e. in the presence of EGTA (Table 5). Besides excluding a major role of calpains by the EGTA experiment this observation is in agreement with the in vitro findings that calmodulin is generally stabilized by Ca^{2+} towards the digestion with e.g. trypsin [53] and thrombin [54].

All results therefore point to an ATP-independent mechanism responsible for the disappearance of calmodulin-conjugates which is sensitive to inhibition by ubiquitin$_t$ (Figs. 5 and 6). The rate of disappearance of conjugates in liver crude extract (Fig. 6C; estimated concentration of ^{125}I-ubiquitin-calmodulin conjugates 5–10 μg/ml) can be estimated from the half-life of 15–20 min to be ca. 3%/(min $\times$ mg). This compares to an over 700-fold lower ATP-independent degradation rate of ^{125}I-BSA in liver crude extract (0.20 arb. unit/mg, Table 2) corresponding to a disappearance rate of ca. 0.004%/(min/mg). Since ^{125}I-BH-calmodulin in the absence of ATP is degraded 2-fold slower than ^{125}I-BSA in reticulocyte lysate (Table 5) it can be estimated that the decay of calmodulin conjugates in liver APF II is at least 3 orders of magnitudes higher than the proteolysis rates of the unconjugated calmodulin moiety alone. This latter very slow proteolysis of calmodulin could be due to the 20S multicatalytic endopeptidase complex activity which can also be strongly influenced by substrate methylation as shown for BSA and concluded for calmodulin [55,56]. No inhibition of the purified 20S multicatalytic endopeptidase complex (generous gift of Dr. L. Kuehn, Düsseldorf) by ubiquitin could be detected in the fluorogenic peptide assay (not shown).

In direct experiments employing ubiquitin-^{125}I-BH-calmodulin conjugates the question as to what reaction leads to conjugate decay could be clarified. It could be demonstrated (see Fig. 7) that within 10 min ca. 50% of the calmodulin conjugates could be deconjugated by an isopeptidase to the original ^{125}I-BH-calmodulin, the reaction being complete after ca. 40 min. In addition it could be shown that this reaction can be inhibited by ubiquitin. It is concluded

from the work in this paper that calmodulin ubiquitylation is reversible, and apparently does not serve to target calmodulin to proteolysis but serves another biological function [4,57] which remains to be fully clarified.

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